REMARKS

Amendments to the claims have been made to respond to the issues and concerns raised in the Office Action, to clarify aspects in the specification and claims, and to refine claim language. The amendments are believed to be consistent with the disclosure originally filed. The amendments also have been particularly presented to avoid, where applicable, any admission or estoppel, generally, negatively affecting the scope of protection provided by the disclosure and claims of the present application, and also in a manner that avoids prosecution history estoppel, limitation of the scope of equivalences, or the like. Claims 1, 10, 174-183, and 185 have been amended. Claims 2-4, 13-15, 18, 30-164, 168, 171, and 184 have been cancelled. Claims 1, 5-12, 16-29, 165-167, 169-170, 172-183, and 185 remain in the application and are believed in a condition for allowance.

Included with the Applicant's current response is an Information Disclosure Statement. Although the Information Disclosure Statement provides additional information for the Office to consider, such information possibly may be material to patentability and accordingly the Information Disclosure Statement is believed by the Applicant to be the only means to comply with its duties under 37 C.F.R. § 1.56.

The Applicant notes that much of the current Office Action appears to mirror the discussion raised in prior Office Actions in the current case. Moreover, a specific response to the submissions made by the Applicant in its Response and Request for Reconsideration dated January 20, 2005, appear to have been addressed in the current Office Action in the text marked as "Response to Arguments". Accordingly, it is believed that addressing the discussion contained in the "Response to Arguments" will fully respond to the issues raised in the current Office Action.

As a preliminary matter, the Applicant notes that many of the issues and concerns related to the present case present complex and intertwining considerations.

Accordingly, in the event questions remain, the Applicant requests the opportunity to pursue an interview to resolve any issues or concerns.

With respect to the further enablement issues cited in the Office Action, the Applicant disagrees with such enablement concerns raised. In particular, the Office lists a number difficulties associated with successful artificial insemination using low numbers of sorted sperm – for example sperm cell sensitivity, response to different chemical environments, damage to sperm due to sorting, and obtaining sufficient quantities of undamaged sperm – and states that Applicant's prior response now asserts that the means for overcoming these problems are all obvious. However, this is not an accurate characterization of the Applicant's argument. The Applicant has not asserted that overcoming these and other difficulties referenced in the Applicant's application are obvious. Indeed, the Applicant has taken pains to point out that the invention represents significant inventive steps to overcome these issues. Although several embodiments of the invention address these difficulties, as just one example discussed in Applicant's prior response it is noted that chemically coordinating a sheath fluid minimizes stresses upon sperm cells, thereby increasing the successful application of artificial insemination using low numbers of sorted sperm. To reiterate:

> "While naturally it is possible to adjust either the pre- or post-sort fluids, in one embodiment the invention adjusts the sheath fluid (3) so that it imposes significantly less stress upon the cells than was previously accomplished. In one regard the invention is remarkable in that it removes the total focus from that of operation of the flow cytometer to a focus on handling and removing stress from the cells themselves." Specification at page 12, lines 11-16.
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> * * *
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> "For the sheath fluid, a substance is selected according to one embodiment of the invention so that it may be chemically coordinated to present minimal changes. Thus, by selecting the appropriate sheath fluid not only in context of flow cytometry parameters, but rather also in context of the cell parameters themselves, the changes experienced by the cells and the over all result of the sorting can be enhanced. This is shown conceptually in Figure 3. Figure 3 shows some type of chemical factor (such as citrate or other factors) as it may exist throughout the various phases of the process. For instance, the four phases shown might represent the following shown for a flow cytometry separation technique, but not to be so limiting: phase I may represent the existence of the cells within the cell source (1), phase II might show the existence of the cells as they are sorted in the sheath fluid environment, phase III might show the cells as they are collected after sorting and phase IV might show the reconstituted cells in a storage medium after sorting. These four phases as shown for the prior art may experience vastly

> different chemical factor environments. As shown conceptually, however, in the present invention the cells may experience very little change, most notably the dip or drop experienced between phases I and II may be virtually absent. This is as a result of the selection of the appropriate sheath fluid as mentioned above. Thus, as a result of being subjected to an appropriate sheath fluid, the cells in the present invention may experience a much lower level of stress." Specification at page 13, lines 1-18.

Instead, what the Applicant has pointed out as requiring merely routine experimentation is that these inventive principles may be applied to non-bovine species. Recall that the Office's enablement concerns appear to be predicated on the applicability of Applicant's techniques to non-bovine mammals. This distinction between inventive principles and their applicability to a given species of mammal is illustrated even by Applicant's examples relating to bovines. As discussed, in one embodiment an inventive principle is chemically coordinating a sheath fluid. For bovine applications, such a sheath fluid may be a 2.9% sodium citrate solution. In applications to other mammal species, the precise sheath fluid is determinable merely by routine experimentation. That this merely is a matter of routine experimentation is exemplified for example at least by a review of the prior art supplied to the Office in the Applicant's Information Disclosure Statements. Review of these references illustrates that artificial insemination techniques are well established, as is their applicability to a wide variety of mammals. Indeed, the prior art is replete with specific methods and compounds developed for various mammal species. In this manner, it may be seen that developing species-specific sperm extenders is merely a matter of routine experimentation. What is not taught in this body of prior art is chemically coordinating a sheath fluid – this is an inventive technique of the Applicant, at least for the illustrative embodiment of the invention here being discussed. Moreover, it is the Applicant's inventive techniques, of which chemically coordinating a sheath fluid is just one, that allow the Applicant's invention to achieve the claimed success levels using the claimed numbers of sperm. Accordingly, it may be seen that the application teaches the inventive techniques to overcome the difficulties cited by the Office and differentiates these teachings from the routine experimentation sufficient to apply them to a variety of mammal species.

In addition, the Applicant disagrees that the scope of the claims does not bear a reasonable correlation to the scope of enablement. With respect to achieving at least 90%

success rates, please find attached to this response as Exhibit A a publication by an inventor listed in the current case, Schenk, John L., "Applying Sperm Sexing Technology to the AI Industry", Proceedings of the 18th Technical Conference on Artificial Insemination & Reproduction (2000). This publication provides evidence demonstrating that the specification of the current case enables the scope of the recitation in the claims of "establishing an insemination sample capable of fertilizing at least one egg within said female of said nonhuman species of said mammal at success levels selected from the group consisting of at least 35%, at least 41%, at least 50%, and at least 90% of a typical insemination dosage and having a number of separated nonhuman sperm cells less than about one-half the number of sperm cells of said typical insemination dosage". The Schenk publication at page 74 notes that pregnancy rates for artificially inseminated beef females are in the range of 50%-60% per insemination. In fact, the true average for such pregnancy rates may be closer to 50% than 60%. More to the point, data discussed in the Schenk publication at Tables 1 and 2 demonstrate the achievement of 54% average pregnancy rates for the Colorado trials and 53% average pregnancy rates for the Nebraska trials, each conducted with a low dose of 1.5 million sexed sperm. These pregnancy rates support the "at least 90%" value recited by the claim and in fact achieve and even exceed 100% of the true average for pregnancy rates achieved using typical artificial insemination. Importantly, the techniques used in the Schenk publication are not significantly different from the techniques taught by the current case. In this manner, it may be seen that the teachings of the specification of the current case not only enable the claimed success rates, but that such claimed success rates were actually experimentally achieved.

The Office cites the results of Cran to argue the unpredictability of achieving the Applicant's success rates. However, the Office has not pointed out if or how Cran uses the techniques taught by the Applicant's application, nor has pointed out why Cran should be cited against the Applicant in light of the deficiencies of Cran noted even by the Office related to delay between semen collection and insemination, asynchrony between insemination and ovulation, semen doe and the onset of estrous.

Further, the Applicant disagrees that the specification must exemplify a representative number of sheath fluids that can be used with any mammalian sperm. As previously discussed, an inventive principle for this embodiment of the invention is not the selection of particular sheath fluid compositions for individual mammalian species. Such selection may be accomplished through routine experimentation, as previously discussed. Rather, as discussed in the specification at page 6, lines 15-19, the relevant inventive principle is that of replacing the sheath fluid as typically was used in flow cytometers at the time of the invention with a sheath fluid which minimizes the stress on sperm cells as they are sorted. This principle was not known in the art prior to the Applicant's invention and represents a significant improvement in the ability to sort sperm that is applicable to a broad variety of mammalian species. Because the Applicant first discovered this principle, the Applicant is entitled to claim its full breadth across the variety of mammalian species to which it may be applicable.

The Office states the specification has not in fact taught how the extensive number of factors and parameters encompassed by the invention will affect the claimed subject matter. However, this assertion misconstrues the nature of the Applicant's argument. The Applicant's point that there is predictability in the art if one can anticipate how a change will affect the claimed invention was made only with respect to adjusting the parameters of a flow cytometer. Recall that the prior Office Action raised this issue only with respect to claims 172-173 and 182-183. Accordingly, the Applicant's prior response was addressed to the scope of the issue raised. Moreover, the Applicant's prior response illustrated that the ability to sort sperm is preferably provided through flow cytometry, as described in the specification at page 8, lines 21-22, and that it is the adjustment of the parameters of a flow cytometer that are predictable. To reiterate, the use of flow cytometers to sort various types of cells, including sperm cells, is well known in the art. Indeed, many of the references supplied by the Applicant via Information Disclosure Statement and incorporated by reference in the present case expressly discuss various methods and apparatus related to the use of flow cytometers. As readily may be appreciated, a flow cytometer is a mechanical device having parameters which may be adjusted to direct its performance. It is well known that the accuracy of the sorting

percentages achieved by a flow cytometer can be predictably varied by adjusting the parameters of the flow cytometer. As just one example, reducing the sort rate of a flow cytometer predictably increases the accuracy of the sorting percentages achieved by the flow cytometer. In this way, persons skilled in the use of flow cytometers readily may anticipate the effects that changing the parameters of the flow cytometer may have on sorting accuracy. While a degree of experimentation may be required in any given situation to adjust the parameters of the flow cytometer to achieve a desired accuracy of sorting percentages, such experimentation is not atypical in the field of flow cytometry and in any case is facilitated by the predictability of making adjustments to the flow cytometer. For these reasons, the Applicant notes that the experimentation required to adjust a flow cytometer to achieve a desired accuracy of sorting percentages is not undue.

With respect to the Office's emphasis on Applicant's Example 1, the Applicant disagrees that Example 1 is the only portion of the specification that may provide enablement for the claims. Importantly, it is incumbent upon the Office when assessing enablement to view the specification as a whole. When analyzing the enabled scope of a claim, the teachings of the specification must not be ignored because claims are to be given their broadest reasonable interpretation that is consistent with the specification. MPEP § 2164.08. One does not look to the claims but to the specification to find out how to practice the claimed invention. MPEP § 2164.08; *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 721 F.2d 1540, 1558 (Fed. Cir. 1983); *In re Johnson*, 558 F.2d 1008, 1017 (CCPA 1977). Here, as has been discussed, Example 1 merely is one practical application of the more general inventive techniques discussed throughout the specification as a whole. The specification teaches the principles underlying a variety of inventive techniques that, taken individually or in combination, produce the results claimed by the Applicant. By way of illustration, and as has been discussed, just one example of these inventive techniques is chemically coordinating a sheath fluid. Moreover, even with respect to Example 1 itself, the Applicant has shown in remarks made above and in its prior response that the scope of enablement provided by Example 1 is reasonably correlated to the scope of the claims, because the specification by way of Example 1 discloses at least one method for making and using the claimed invention that

bears a reasonable correlation to the entire scope of the claim, MPEP § 2164.01(b); *In re Fisher*, 427 F.2d 833, 839 (CCPA 1970). In particular, Example 1 illustrates a practical application of the inventive principles more generally taught in the specification – again, for example, the general inventive principle of selecting a sheath fluid that is chemically coordinated to a pre-sort and a post-sort chemical environment is embodied in the example by the use of 2.9% sodium citrate in the sheath fluid for bovine applications. With an understanding of the relevant inventive principles, an artisan is able to apply these principles to other mammalian species through routine experimentation, as discussed above and in the Applicant's prior response.

The Office raises further indefiniteness concerns under 35 U.S.C. § 112. With respect to the recitation of a "typical insemination dosage," the Applicant disagrees that if the artisan needs to perform an experiment in order to determine the typical dosage, then clearly what constitutes a typical dosage is not known and defined in the art. Even assuming as correct the Office's definition of "typical" as meaning what is commonly encountered or the average, there is no prohibition against conducting experimentation to establish typical values. To the contrary, a significant degree of experimentation is permissible, so long as such experimentation is merely routine. To reiterate from Applicant's prior response, the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. MPEP § 2164.01; *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). An extended period of experimentation may not be undue if the skilled artisan is given sufficient direction or guidance. MPEP § 2164.06; *In re Colianni*, 561 F.2d 220, 224 (CCPA 1977). The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. MPEP § 2164.06; *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988) (citing *In re Angstadt*, 537 F.2d 489, 502-04 (CCPA 1976)). Moreover, for the variety of mammals routinely subject to artificial insemination, the typical insemination dosage in fact already

may be known as a result of the very type of routine experimentation described. For example, as pointed out in the specification at page 19, lines 12-14, for bovines an absolute number of 500,000 sperm may be considered a low dose where currently 1 to 10 million sperm are provided. Accordingly, the typical insemination dosage indeed may be known in the art either as an actual value for certain mammalian species or through well-established techniques of routine experimentation for other mammalian species.

With respect to the recitation of "sensing a sex characteristic", the Applicant disagrees that this term is indefinite. However, the Applicant notes the Office states it is unclear as to whether sensing encompasses actually determining a sex characteristic. While the Applicant disagrees that the term "sensing" is unclear in this manner, to facilitate examination the Applicant has amended the claims to recite the term "determining a sex characteristic".

With respect to the recitation of a "time which is generally regarded as optimal", the Applicant disagrees that this term is indefinite. However, the Applicant notes the Office states that its concern is based not on the fact that that one of skill in the art would not know how to perform an experiment to determine a time at which insemination may occur, but rather that there is no art-recognized definition for the term. While the Applicant disagrees that there is no art-recognized definition for the term, to facilitate examination the Applicant has amended the claims to recite the term "an optimal time for a single insemination".

With respect to the recitation of "separating nonhuman sperm cells based upon said sex characteristic and a rate of at least 1200 sorts per second", the Applicant disagrees that this term is indefinite. However, to facilitate examination the Applicant has amended the claims to recite the term "separating nonhuman sperm cells based upon said sex characteristic and a rate of at least 1200 separations per second".

The Office expresses further obviousness concerns with respect to the combination of Seidel (1996) and Rens. However, in its Response to Arguments, the

Office appears to mingle two issues that were separately raised in the prior Office Action – Applicant's recitation of 1200 sorts per second (now amended to 1200 separations per second) and Applicant's recitation of a collection container.

For example, the Office states that Applicant's response appears to indicate that the asserted levels of sperm dosages and success rates obtained by sorting at 1200 sorts per second can only be achieved using the special collection device discussed at pages 24-28 of the specification. However, this incorrectly states the Applicant's argument. The Applicant has not asserted that such a collection device is critical in the manner attributed to it by the Office. Indeed, as previously discussed, the Applicant has repeatedly stressed that the invention contains several aspects in various embodiments that, alone or in combination, may allow the claimed results to be achieved. An example of just one such other aspect is the teaching of chemically coordinating a sheath fluid, as previously discussed. Instead, with regard to a collection container, the Applicant's prior response merely pointed out that the combination of Seidel (1996) and Rens cited by the Office does not teach the collection containers described and claimed by the Applicant. In particular, the Office used this combination to support its obviousness concern by citing Rens at column 3. However, the Office does not appear to have addressed Applicant's notice in its prior response that Rens at column 3 does not discuss collection containers, but rather only the dimensions of a nozzle in order to properly orient sperm. As stated in Applicant's prior response, this is significantly different from the Applicant's claims, which recite the dimensions of a collection container in order to reduce damage to sperm cells. Accordingly, the current case identifies an entirely different problem than that of Rens and solves it in an entirely different manner than that of Rens. Moreover, the Office states that the Applicant argues that merely because the solution to a substantial problem appears simple in hindsight does not make it obvious. Again, the Applicant notes that this statement was made to address the Office's obviousness concern with respect to collection containers, and in particular to address the Office's assertion that it would have been well within the skill of the art at the time the invention was made to have selected a collection container of an appropriate width in order to have prevented damaging sperm since Rens does teach the criticality of the of the dimensions of the

sorting device and the orientation of sperm within the sorting device in order to maintain sperm viability. However, a statement that modifications of the prior art to meet the claimed invention would have been "'well within the ordinary skill of the art at the time the claimed invention was made'" because the references relied upon teach that all aspects of the claimed invention were individually known in the art is not sufficient to establish a *prima facie* case of obviousness without some objective reason to combine the teachings of the references. MPEP § 2143.01; *Ex parte Levengood*, 28 U.S.P.Q.2d 1300 (Bd. Pat. App. & Inter. 1993). Accordingly, it is the Office's responsibility to provide an objective reason for combining the references beyond merely that aspects of the claimed invention may have been individually known. As discussed, the teachings of Rens and the Applicant's invention relate to entirely separate aspects of sorting devices (nozzle dimensions of the sorting device itself in Rens, versus dimensions of a collection container in Applicant's invention) that are directed to entirely different purposes (orienting sperm in Rens, versus collecting sperm to minimize damage by impact with a container in the Applicant's invention). The Office has not provided an objective reason as to why the ordinary artisan would have combined the references in the manner suggested.

On the issue of Applicant's recitation of 1200 sorts per second (now amended to 1200 separations per second), the Applicant again has merely pointed out the combination of Seidel (1996) and Rens does not teach 1200 sorts per second. In this regard, it is important to realize the difference between the sample rate taught by Rens and the sort rate taught by the Applicant. Importantly, the Office in its Response to Arguments does not appear to address this distinction pointed out by the Applicant in its prior response. As discussed there, a sample rate refers merely to the number of fluorescent events analyzed each second, whereas a sort rate refers to the actual number of sperm sorted each second. There are less sperm sorted each second than are analyzed because not every analysis event yields a conclusion certain enough to warrant a sort. As stated in Rens at column 4, line 17, "the elliptical nozzle of this invention is capable of orienting in excess of 60% of sperm for sorting." This means that only about 60% of the sperm sampled in Rens are actually sorted. As further discussed in Rens at column 2,

lines 4-10, as many as 60%-80% of sampled sperm detected by other processes are not sorted. Consequently, regardless of the sample rate values discussed by Rens, at best only about 60% of the number of sampled sperm are actually sorted. Further, this 60% value is a best-case figure, and Rens does not discuss for which particular sample rate the 60% value was achieved nor the parameters required to achieve the same. In fact, Rens does not discuss any actual sort rates achieved at all. In this manner, it may be seen that Rens in fact does not teach the sort rates taught by the Applicant, and its combination with Seidel (1996) fails on this point. Moreover, the Office states that in the absence of evidence to the contrary, methodologies known in the art for protecting sorted sperm and the methodologies utilized by Seidel (1996) and Rens are expected to have been sufficient in order to have allowed the artisan to have practiced the sorting method at rates of 1200 sorts per second. However, as evidence the Applicant points to Seidel (1996) and Rens themselves, neither of which teach achieving 1200 sorts per second. As a result, the methodologies of these references clearly cannot be presumed sufficient to have allowed the artisan to achieve 1200 sorts per second, as is suggested by the Office, and the burden is on the Office to provide evidence to the contrary.

The Office expresses further obviousness concerns with respect to the combination of Seidel (1996), Rens, and Seidel (1994). However, the combination of Seidel (1996) and Rens fails for the reasons discussed above and therefore cannot be combined with Seidel (1994) to support the obviousness concerns raised by the Office.

The Office expresses further obviousness concerns with respect to the combination of Seidel (1996), Rens, Rath, and Seidel (1995). However, the combination of Seidel (1996) and Rens fails for the reasons discussed above and therefore cannot be combined with Rath and Seidel (1995) to support the obviousness concerns raised by the Office.

The Office raises several concerns related to the Seidel (1997) reference. The Applicant believes this reference may be properly removed pursuant to the terms of MPEP § 715.01(c). However, the Office has cited certain issues with respect to the

affidavit provided by the Applicant in its prior response. In order to more accurately ascertain the information desired by the Office for such an affidavit, the Applicant anticipates discussing the content of such an affidavit by way of an interview.

The Office has raised several nonstatutory double patenting concerns. Please find attached to this response as Exhibit B a terminal disclaimer that the Applicant believes is sufficient to overcome these concerns.

The Applicant, having addressed each of the concerns raised in the Office Action, respectfully requests reconsideration and withdrawal of the rejections and objections to the application. Allowance of claims 1, 5-12, 16-29, 165-167, 169-170, 172-183, and 185 is requested at the Office's earliest convenience.

Dated this 20 day of October, 2005.

Respectfully submitted,
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By: _____

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EXHIBIT A

APPLYING SPERM SEXING TECHNOLOGY TO THE AI INDUSTRY

John L. Schenk
XY, Inc.
Ft. Collins, CO

SCHENK

John L. Schenk, serves as Manager of Sperm Processing and Flow Cytometry Laboratories for XY, Inc., a company that is focused on developing sperm sorting technologies. He has spent his entire career in the field of cattle physiology and reproduction. Schenk holds a B.S. degree in Bio-Agricultural science as well as a M.S. degree in Reproductive Physiology from Colorado State University. His experience includes nearly eight years as a Reproductive Physiologist for ABS Global, four years in production and customer service with Ankony Shadow Isle and Colorado State University as Manager of Stud Operations.

Radical change in the artificial insemination (AI) industry during the past 10 years has caused genetic companies and cattle producers alike to search for opportunities to increase earnings. Narrow profit margins for genetic companies and producers, mergers and strategic alliances of multiple genetic companies, and the ever-expanding global market are transforming markets. Competition among genetic companies mandates rapid identification of elite sires. Genetic company profits are realized through optimizing sperm harvest of bulls, prudent packaging of sperm to maintain maximum fertility, and marketing products that meet consumer expectations. Sex-selected sperm will allow production of offspring of the desired sex from special matings to take advantage of differences in the value of males and females for specific markets. Genetic companies must develop operational and marketing strategies now for the implementation of sex-selected sperm.

One of the sexes usually is a byproduct in specified production systems. Bulls and heifers are sexually dimorphic in function, and commercial value often differs as well. Numerous traits important to dairy and beef cattle production create the opportunity for commercial exploitation of sex-selected sperm. Synergistic uses of sex-selected sperm with other assisted reproductive technologies (ARTs) like AI, in-vitro fertilization (IVF) and embryo transfer create opportunities that will increase production efficiencies.

A European genetic company has recently introduced sex-selected bovine sperm commercially. This eagerly awaited event plus increased producer demand will pressure other genetic companies to provide sex-selected sperm to their customers, or risk losing market share. At this time, widespread use of sex-specific sperm for AI is precluded by limitations in current sperm sex separation technology. As with other new technological products, initial production and purchase costs are high, but as efficiencies are increased and improved technologies implemented, associated sex-selected sperm costs will be reduced.

Pre-determination of offspring sex will increase economic efficiencies and hasten genetic progress. For genetic companies, the increased rate of response to selection will reduce costs of achieving genetic change, and hence, potentially increase profits. Herein, I'll describe state-of-the-art sperm sorting technology using a flow cytometer/sperm sorter (flow-sorting) and its potential impact on operations and profitability of genetic

companies. Applications of sperm sorting technology to cattle production (5) and commercial economic issues (1) have been described elsewhere.

Development and Licensing of Flow-sorting Technology

Flow-sorting technology for viable sperm was originally developed by USDA researchers in Beltsville, MD, and patented by the United States government. The patent requires payments of royalties to the United States government upon commercialization of the technology. Exclusive rights to the patent, for all but human uses, have been assigned to XY, Inc. XY, Inc. and researchers at Colorado State University have focused on developing low cost, low maintenance, reliable flow-sorting technology specifically for sperm. Procedures for freezing sorted sperm and novel methods to inseminate females with fewer sperm than used in conventional AI continue to be investigated.

XY, Inc. intends to sub-license genetic companies to use state-of-the-art sperm-sorting technology. The sorters themselves currently cost $280,000 each, although this cost should decline markedly when the sperm sorters are simplified and mass-produced. Since freshly collected semen is required for optimized sorting, sorters likely will be placed at major bull studs for actual production and distribution of flow-sorted sperm.

Principles of Flow-sorting Sperm

The probability of obtaining a particular sex of offspring from any mating is close to 50%. Presently, flow-sorting viable X-chromosome and Y-chromosome-bearing sperm is the only proven, reliable method that has resulted in the birth of a preponderance of desired-sex offspring (6). Bovine X-chromosome-bearing sperm (female) contain 3.8% more DNA than Y-chromosome-bearing sperm. This is the basis for separating sperm via flow-cytometry/sperm sorting.

Freshly collected sperm stained with Hoechst 33342, a DNA-specific binding dye, fluoresce when excited by light of ~360 nm wavelength. A beam of laser light intersects a pressurized fine stream transporting sperm. Since X-chromosome-bearing sperm contain more DNA, they bind more Hoechst 33342 than Y-chromosome-bearing sperm, and thereby emit a brighter signal when exposed to laser light. Properly positioned detectors on the sperm sorter quantify the intensity of light emitted from each sperm and transfer that information to a computer for processing. Due to the asymmetrical morphology of the sperm head, fluorescence is detected most accurately when the light beam intersects the flat surface of the sperm. Sperm are oriented with ~70% accuracy by manipulating the shape of the stream as it passes the detectors. During sperm transit through the sorter, a vibrating crystal breaks the stream into small droplets, of which ~30% contain sperm. A positive or negative electrical charge is assigned to droplets according to the brightness of each sperm. Droplets then pass through an electric field where opposite charges cause the deflection of sperm-containing droplets. Droplets containing improperly positioned sperm are not charged and collected into a waste container. Streams of droplets containing selected sperm are collected into a test tube for further processing. Only live, membrane-intact sperm are collected; dead sperm and even some sperm with chromosomal aberrations are removed during flow-sorting.

A modular flow cytometer/sperm sorter (MoFlo® SX, Cytomation Inc., Fort Collins, CO) is capable of producing nearly 90,000 drops/sec. Currently, sperm sorting is performed at ~25,000 events/sec, an event being a droplet containing a sperm. Approximately 15 x 10^6 live sperm of each sex at 90% accuracy currently can be sorted per hour, the rate depending on characteristics of individual ejaculates.

Purity of sorted sperm is confirmed by resort analysis, a quality control procedure that determines the actual purity achieved. Incorporation of this assay is mandatory and minimizes disappointment by the end user (14). Total sperm recovery during flow-sorting is approximately 30% of the original sperm number, if both X- and Y-chromosome-bearing sperm are collected. It would be impractical to sort all doses of semen produced at current sort rates, but for certain high profile markets and niche ARTs like IVF-ET, flow-sorted sperm likely will be applied as soon as this product is available.

Market Potential and Applications

USDA estimates for the number of dairy cows and heifers, and beef cows and heifers that have ever calved was 9.3 and 34.8 million head, respectively in 1998 (13). Based on the 1999 National Association of Animal Breeders semen sales report for the United States (8), approximately 2.4 doses of semen are used for each dairy female to become pregnant; about 60% of the dairy population is bred AI. Pregnancy rates for beef females are in the range of 50 to 60% per insemination, and ~5% of the beef females are inseminated by AI. Heifer replacements for dairy and beef herds are relatively few compared to the number of cows in production. Therefore, the largest sex-selected sperm market to target would be for cows. Unfortunately at the present time, flow-sorted sperm are only recommended for use in heifers, which become pregnant more readily with lower sperm numbers per insemination dose than do cows.

Dairy Bull Production

The dairy industry is driven by production of milk and milk components. Using semen from proven, genetically superior sires for decades is primarily responsible for increased genetic gains within the dairy industry. Identifying genetically superior bulls is time-consuming and expensive. Elite matings between genetically superior dams and sires are designed to produce bulls. Unfortunately, half of these pregnancies result in the birth of a heifer, and sex-selected sperm could make these matings more efficient.

Progeny testing young bulls for dairy traits with 85% reliability requires 80 daughters that complete a first lactation in 60 herds. In 1975, six inseminations with non-sorted sperm were necessary to produce a daughter completing a first lactation (4). Today, lower herd fertility, incomplete daughter reporting systems, and fewer inseminations by genetic company trained-inseminators and more done by dairymen contribute to an increase in the number of inseminations per lactating daughter to 10 or more. Using the model put forth in 1975, a sex-selected product enriched for the X-chromosome bearing sperm would reduce the number of inseminations required to produce a female to 3.3 provided all other things are equal. The cost to produce an informative heifer would thus be reduced. Rapid production of daughters with a greater distribution within and across herds from more progeny test bulls could be achieved. Genetic progress also would be hastened with the use of sex-selected sperm in a progeny test program because fewer highly selected dams would be required to produce the bulls.

Multiple Ovulation and Embryo Transfer (MOET)

MOET increases the probability of producing at least one bull without the use of sex-specific sperm. Slight statistical and economic gains are realized when sex-specific sperm are used in MOET programs. The probability of producing three live offspring with at least one of them being the desired sex is increased from .88 with non-sorted sperm to .99 when inseminations containing sperm that are 90% of the desired sex (5). Fewer embryo transfers would be required to achieve the same number of desired sex offspring.

Breeding Heifers to Have Heifers

Heifers on the average are genetically superior to the base cowherd. Replacement heifers bred to X-enriched sperm will allow producers to take advantage of genetic superiority, decrease the generation interval, and all other factors being equal, increase the rate of genetic improvement. Calf birth weight is the most important variable affecting dystocia. Heifer calves tend to be smaller at birth than bulls, minimizing dystocia. Mating virgin heifers to X-chromosome-bearing sperm will reduce dystocia (2) and increase calf survival (7). Other factors that are economically affected by dystocia include: increased postpartum interval, increased days open, decreased conception rate, decreased milk production and increased retained placentas, cow mortality, veterinary and labor costs. X-chromosome-bearing sperm for use predominately in heifers also has the potential to increase the number of beef heifers bred with AI. Sex-selected sperm for use in heifers may be the incentive the AI industry has been searching for to increase numbers of beef cattle inseminated artificially.

Increased Milk and Beef Production

Matings between elite dairy sires and specialized high component dams would produce fertile crossbred heifers. Net profit from these lactating heifers is increased because of higher milk components (% protein and % butterfat). Additionally, genetically average-milking cows mated with Y-bearing sperm from beef sires would lead to efficient dairy-beef production.

Commercial beef producers will be able to make more efficient use of heterosis in crossbreeding systems. F-1 females with high maternal characteristics for fertility, milking and mothering ability would be mated to terminal cross sires with desirable traits for rapid, efficient and high growth rates. Hybrid vigor will be at the highest possible level utilizing maternal, and growth traits to maximize calf weights. For meat production, steers grow more rapidly and efficiently than heifers, and produce a more valuable carcass. Y-chromosome-bearing sperm from sires transmitting genetic traits such as increased growth rate, feed efficiency, muscling, marbling and carcass quality will be used to produce steers for beef consumption.

Fertility of Flow-sorted Sperm

Field trials to determine how well flow-sorted sperm perform under farm conditions are continuing. Critical numbers of flow-sorted sperm have been inseminated, so that beneficial sperm treatments and effects of flow-sorting could be studied (12). In these trials, 1 to 3 x 10^6 flow-sorted total sperm from 22 bulls of unknown fertility, but selected for acceptable semen qualities were inseminated into 1000 heifers. Control unsexed inseminates (n = 370) containing 20 x 10^6 total sperm served as a composite estimate of the intrinsic, normal fertility of the heifers within studies as well as for bull fertility and inseminator skill. The 2-month pregnancy rates determined by ultrasound were 47% (29% to 86%) for

determined by ultrasound were 47% (29% to 86%) for flow-sorted sperm and 63% (33% to 78%) for controls. No excess abortions were found between 1 and 2-months of gestation; losses were 23 of 261 (8.8%) for sexed pregnancies and 9 or 145 (6.2%) for controls. Actual sexes of fetuses and calves over all studies were 86% of the desired sex. Both heifers and bulls were produced accurately, with no gross abnormalities observed.

Recently, three additional trials inseminating heifers with flow-sorted frozen/thawed sperm have been conducted (Tables 1, 2, 3); 531 heifers were inseminated with flow-sorted sperm (1.5 to 6 x 10^6 total sperm/dose), along with 278 heifers with unsexed control sperm (20 x 10^6 total sperm/dose). In all studies, heifers were synchronized, visually inspected mornings and evenings for estrus, but inseminated once a day, 12 h or 24 h after onset of estrus. Insemination was either into the uterine body conventionally, or in the case of field trial 13 (Table 2), one half of the sorted sperm inseminated were deposited into the uterine horn that was first encountered and the remaining inseminate into the uterine body using atraumatic embryo transfer sheaths (IMV, Minneapolis, MN). Semen was deposited past the greater curvature of the uterine horn as far anterior as could be accomplished without trauma, identically to nonsurgical embryo transfer. The 2-month pregnancy rates determined by ultra-sound were 53% (31% to 69%) for flow-sorted sperm and 68% (61% to 77%) for unsexed controls.

Table 1. Results of field trial 12 — Angus heifers pregnant (%) in Colorado.

Treatment/Site	Bulls		Average
	AN012	AN013	
20 x 10^6 Unsexed – Body	45/64 = 70	40/62 = 64	85/126 = 67[a]
4.5 x 10^6 Sexed – Body	26/55 = 47	36/67 = 54	62/122 = 51[b]
1.5 x 10^6 Sexed – Body	32/60 = 53	35/63 = 56	67/123 = 54[b]

[a,b] Means without common superscripts differ (P<.05).
Control sex ratio: 44% female fetuses.
Sorted sex ratio: 95% of that predicted.

Table 2. Results of field trial 13 — Red Angus heifers pregnant (%) in Nebraska.

Treatment/Site	Bulls		Average
	AR002	AR004	
20 x 10^6 Unsexed – Body	34/56 = 61	41/56 = 73	75/112 = 67[a]
1.5 x 10^6 Sexed – Horn	29/51 = 57	28/55 = 51	57/106 = 54[b]
1.5 x 10^6 Sexed – Horn	28/51 = 55	28/54 = 52	56/105 = 53[b]

[a,b] Means without common superscripts differ (P<.05).
Control sex ratio: 49% female fetuses.
Sorted sex ratio: 92% female fetuses.

Fertility of low-dose flow-sorted sperm varies, depending on the exact conditions of use. Pregnancy rates with flow-sorted sperm have been 70 to 90% of those obtained with unsexed controls. Improved procedures for the use of flow-sorted sperm should stabilize results. Huge differences exist among bulls regarding pregnancy rates at low dosages of unsexed sperm (3). It is likely that similar fertility trends will apply to flow-sorted sperm for each bull-fertility-group. Identification of the potential sperm fertilizing ability for every bull will be mandatory when critical numbers of flow-sorted sperm are inseminated. Heterospermic insemination might allow rapid determination of bulls of particularly low or high fertility (10).

Table 3. Results of field trial 14 — Angus heifers pregnant (%) in Wyoming.

Treatment/ Site	Bulls			Average
	AN016	AN018	AN019	
20 x 10^6 Unsexed–Body	9/13 = 69	10/14 = 71	10/13 = 77	29/40 = 72[a,b]
6.0 x 10^6 Sexed–Body	5/12 = 42	7/12 = 58	9/13 = 69	21/37 = 57[b,c]
2.0 x 10^6 Sexed–Body	4/13 = 31	8/12 = 67	6/13 = 46	18/38 = 47[c]

[a,b,c] Means without common superscripts differ (P<.05).
Control sex ratio: 46% female fetuses.
Sorted sex ratio: 85% female fetuses.

No statistical difference in pregnancy rates has been found when inseminating heifers with 1.5 to 4.5 x 10^6 total sorted sperm (12). Den Daas et al. (3), found a high correlation between total unsorted sperm inseminated and fertility. Perhaps the differences in dosages of flow-sorted sperm used to date have been too small to detect larger differences in fertility. Little fertility information was available on the bulls used, and only 20% of AI stud bulls are of high fertility at low doses of unsexed sperm (3). We presume that average fertility bulls were used in the studies with sexed sperm; perhaps this fertility at low doses of sperm is too low relative to maximum potential fertility. Seidel et al. (11) inseminated heifers with as few as 500,000 total non-sorted sperm from highly fertile bulls, and compared 2-month pregnancy rates to that for 10 x 10^6 total sperm. There was no significant difference in pregnancy rates at either dosage (least squares means; 66% vs. 64%, respectively).

Insemination of flow-sorted sperm from bulls of high fertility into well-managed heifers may achieve maximum pregnancy rates at dosages around 2 x 10^6 total sperm. Average fertility bulls likely will require at least 6 to 8 x 10^6 total sperm/dose to maintain maximum pregnancy rates. Bulls of low fertility should never be packaged at low dosages, and most certainly would not be good candidates for flow-sorting, simply because sorter and sperm efficiencies would greatly be reduced.

Potential Flow-sorting Damage

Some sperm are damaged before, during and after flow-sorting. Research is continuing to determine precisely which processes involved with flow-sorting cause

the most damage to sperm. For perspective, damage due to sorting is minor relative to that caused by cryopreserving sperm. The propensity of sperm to survive sorting will probably vary similarly to that for successful cryopreservation, because bulls have not been selected based on tolerance of their sperm to sorting. Facets of sperm handling that are being addressed include: effects of high dilution; osmolality and pH of buffers; concentration of stain for DNA; staining and sorting temperatures; effects of laser intensity; centrifugation time and g-force to concentrate sorted sperm, freezing medium, cooling rates; interval between cooling and freezing; and the concentration of sperm during freezing. Laboratory assays used to determine sperm damage are probably too crude to detect subtle differences in flow-sorted sperm quality. Sensitive sperm assays to quantify sperm quality may eventually be used to cull batches of sorted sperm that are most damaged.

Applying Flow-sorting Sperm at Genetic Companies

Initially sex-selected sperm will be recommended only for semen from a few highly fertile bulls for use in heifers. The ease of producing flow-sorted sperm will depend upon the degree sex ratio is altered, bull age and collection frequency, sperm output and quality, availability of labor, and costs of production. Scheduling and operations will differ considerably from those currently employed at genetic companies. The process of negotiating license fees, purchasing and installing sperm sorters, training personnel, and producing and marketing flow-sorted sperm will require considerable investments in time and money. Maximizing related sperm sorter investments will require a paradigm shift in sperm processsing. Flow-sorting sperm is a time-intensive process, and the sorters themselves require supervision to perform for extended periods of time. Collection of sufficient high quality sperm for processing requires hours of sorting in addition to the time required for novel processing methods before the sperm are frozen. Supplementary labor will be required to maximize sorter efficiencies. Assurance of the best possible sperm, post-sorting, may require collection of ejaculates at different times during the sorting day.

Disadvantages to flow-sorted sperm include possible damage to genetic material, although none has been observed or reported. Uncompensable spermatozoal deficiencies also may exist with flow-sorted sperm. Deficiencies that cause a reduction in pregnancy rates of less than 5 percentage points will be tolerated for many applications. However, decreased pregnancy rates of greater than 5 percentage points would be economically harmful in most situations. We have not ascertained whether pregnancy rates with flow-sorted sperm are lower due to insufficient sperm per insemination dose or because of uncompensable sperm damage. The scope of field trials conducted thus far have not critically examined controls such as non-sorted, stained, highly diluted sperm packaged at low-dosage to accurately assess potential damage to sperm during sorting.

Inseminating large numbers of sperm can often mask low seminal quality. However, for high demand bulls, after information is available for fertility, lower sperm numbers are frequently used provided fertility does not decline noticeably. Low dose packaging will be required for limited, valuable flow-sorted sperm.

Freezing flow-sorted sperm provides flexibility (9), and unfrozen sorted sperm will be limited to regional application. The use of non-frozen sperm is problematic because of the simultaneous need to match sperm sorters with bulls and to have females that are in estrus. However, special situations such as IVF-ET might benefit from the use of non-frozen sorted sperm.

Introducing sex-specific sperm for commercial use will probably require 2 to 5 years. Amann (1) estimated that the current cost of producing and packaging 2×10^6 flow-sorted sperm at $30 to 46. There also may be regulatory constraints to overcome on a global scale. Perhaps special labeling will be required to prevent fraud.

Summary

Flow-sorted sperm to produce sex-selected offspring will not be a panacea for all applications or cattle producers, or for the business of genetic companies. Increased costs of producing flow-sorted sperm from genetically valuable germ plasm, must be offset by increased product costs to the consumer. Initially, these costs may have to be absorbed by the genetic companies unless the product is exclusively marketed to seed stock producers and/or used in house for the production of genetically superior AI bulls.

Effective commercial use of sex-specific sperm will require greater management and labor skills, including well-trained inseminators. Attention to detail will be mandatory, because fewer sex-selected sperm will be used than with conventional AI. The use of sex-selected sperm at low dosage from marginal fertility bulls probably will result in unacceptably lowered conception rates. Furthermore, lower pregnancy rates associated with poorly managed herds, faulty field handling of frozen semen, or improper placement of the semen will be magnified. Genetic company-sponsored educational workshops will be mandatory for end users.

Sex ratio of calves has been altered greater than 85% when flow-sorted sperm have been inseminated into heifers. Pregnancy rates in heifers have been 70 to 90% of unsexed, frozen controls. Future research testing

the efficacy of sorted sperm in cows is warranted. Additional field trials, including low dose unsexed control sperm, are needed to determine to what extent lower pregnancy rates with sorted sperm are a function of sperm damage or simply insufficient sperm numbers needed for maximum pregnancy rates. A proven procedure for sorting sperm by sex has finally been introduced that can be applied to the commercial world. Commercial sex-selected, frozen sperm for AI of heifers in North America should be available within two years.

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